THE EFFECTS OF PERITONEAL DIALYSIS FLUID AND ITS COMPONENTS ON THE DIFFERENTIATION OF WHITE BLOOD CELLS INTO FIBROCYTES

An Honors Fellow Thesis

by

HANNAH ELIZABETH STARKE

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

May 2012

Major: Biology

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Approved by:

Research Advisor: Richard Gomer
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

The Effects of Peritoneal Dialysis Fluid and its Components on the Differentiation of White Blood Cells into Fibrocytes.
(May 2012)

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Kidneys filter out toxins which accumulate in the blood. In order to replace the function of the kidneys in patients with kidney damage, fluid is injected into the abdominal cavity, and after 4-8 hours, during which the fluid has collected waste products, the fluid is removed. This process is called peritoneal dialysis or PD. Though effective in detoxification, PD can come with a grave side effect: the formation of scar tissue in the abdomen. To form this scar tissue, white blood cells leave the blood and develop into cells called fibrocytes. A blood protein called Serum Amyloid P (SAP) inhibits the development of fibrocytes. I investigated how the dialysis fluid and its separate salt components affect the development of fibrocytes. I found that dialysis fluid and its component salts sodium chloride and sodium lactate all caused significant increases in human fibrocyte development in tissue culture. I then tested the effect of PD fluid and its components on the ability of SAP to inhibit fibrocyte development. I found that whole PD fluid and sodium lactate decreased the ability of SAP to inhibit the

development of fibrocytes. These results show that components of PD fluid enhance the development of fibrocytes, which could provide an explanation for the development of scar tissue during dialysis treatment.

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DEDICATION

This thesis is dedicated to my parents who have never ceased to amaze me in their dedication to their family and their careers. They have never let a day go by without showing me how much they support me in every way possible. Everything I have accomplished is because of them. I love you both so much.

And to my brothers who are always there to help me believe that I can achieve anything and everything. Who make sure that I know they are there for me until the end of days. Matty and Natey, I love y'all so much.

And to everyone else who helped support me and dealt with me throughout this whole experience. I wouldn't have been able to do it without you.

ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude to Dr. Richard Gomer for all of his support and direction that he showed me throughout this whole process. Without his kind words and subtle urging, I would not have been able to accomplish any of the things I have.

I would also like to thank Nehemiah Cox and Michael White for all of their time and patience with teaching, supporting, and guiding me. Without them, this would not have been possible.

Thank you to the rest of the Gomer lab for their acceptance of me and their support for all that I did this year. You all were a great source of encouragement.

NOMENCLATURE

PD	Peritoneal Dialysis
CAPD	Continuous Ambulatory Peritoneal Dialysis
RPMI	Roswell Park Memorial Institute Medium
PBMC	Peripheral Blood Mononuclear Cells
SAP	Serum Amyloid P
GDP	Glucose Degradation Products

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CHAPTER I

INTRODUCTION

Project purpose

This report serves to describe my research. I sought to determine if dialysis fluid and its components enhance the formation of fibrocytes, to understand why scar tissue develops in the abdominal cavity during PD treatment.

Peritoneal dialysis

During late stage kidney diseases, human kidneys no longer perform their function of cleaning toxins out of the blood. In most cases, physicians use dialysis to replace that function [1]. The most common form of dialysis is known as hemodialysis which is performed by running a person's blood through an external machine to clean the blood of waste and biological by-products; however, during late stage kidney failure, continuous ambulatory peritoneal dialysis (CAPD) commonly replaces hemodialysis as a treatment to remove toxins. With CAPD, dialysis fluid is injected into the abdominal cavity via a permanent catheter, and after 4-8 hours, during which it has collected waste products, it is removed and replaced with more fluid [2,3] (Figure 1)

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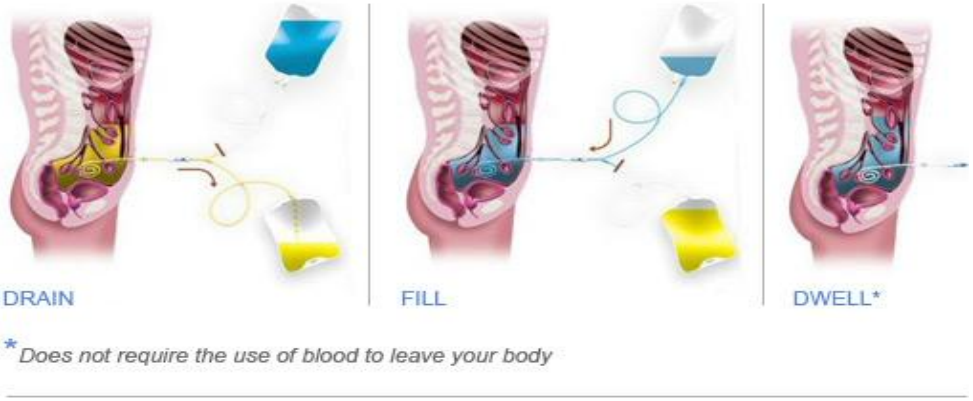

Figure 1: **Schematic of peritoneal dialysis.** Courtesy of Baxter Healthcare Corporation http://www.livenow.info/DiscoverHome/DiscoverPD/PDisDifferent/DifferentProcess.aspx

Peritoneal Dialysis fluids contain a mixture of electrolytes to help maintain blood composition, an osmotic agent to facilitate the transport of water and other liquids through a membrane, and a buffer to maintain proper pH. A commonly used peritoneal dialysis fluid named Dianeal contains the electrolytes sodium, chloride, calcium, and magnesium, the sugar D-glucose as an osmolyte, and a buffer containing lactate [4].

Although this hemodialysis alternative provides less expensive treatment, a more mobile lifestyle, and at-home sessions, one serious drawback exists: the potential to develop a debilitating, deadly disease called peritoneal fibrosis. Although progression into fibrosis occurs in approximately 1% of patients, understanding why fibrosis develops could allow us to develop dialysis fluids that do not cause fibrosis [5]. Glucose degradation products (GDP) (including 5-hydroxymethylfuraldehyde and 3,4-dideoxyglucosone-3-ene) that form during heat sterilization of the PD fluid have an adverse effect and can

promote fibrosis, but researchers have not investigated the effects of other components [6].

Fibrosis and fibrocytes

Fibrosis refers to the formation of unhealthy fibrous scar tissue in an organ or tissue. During an immune response, the body recruits white blood cells to the affected area to repair damage. These cells aid in the destruction of invaders and the formation of scar tissue. However, when the body recruits white blood cells to an area where no injury has occurred, the resulting response can hinder the biological functions of the area [5]. This unnecessary recruitment of fibrocytes can begin the development of a fibrosing disease. Common fibrosing diseases include heart disease, liver cirrhosis, scleroderma, and Crohn's Disease along with many other well known autoimmune diseases. These diseases can lead to the degradation of an organ or tissue system and eventual death of the individual [7].

Fibrosis is characterized by the presence of white blood cell-derived cells called fibrocytes. Fibrocytes promote wound healing by aiding in the creation of fibrous scar tissue [8]. They are visually characterized by being long, spindle-shaped cells with an oval-shaped nucleus (Figure 2). Fibrocytes have a combination of blood cells markers and connective tissue cell markers which distinguishes them from other white blood cells [9,10]. These markers include CD45, CD14, CD90, and a variety of collagen markers [9].

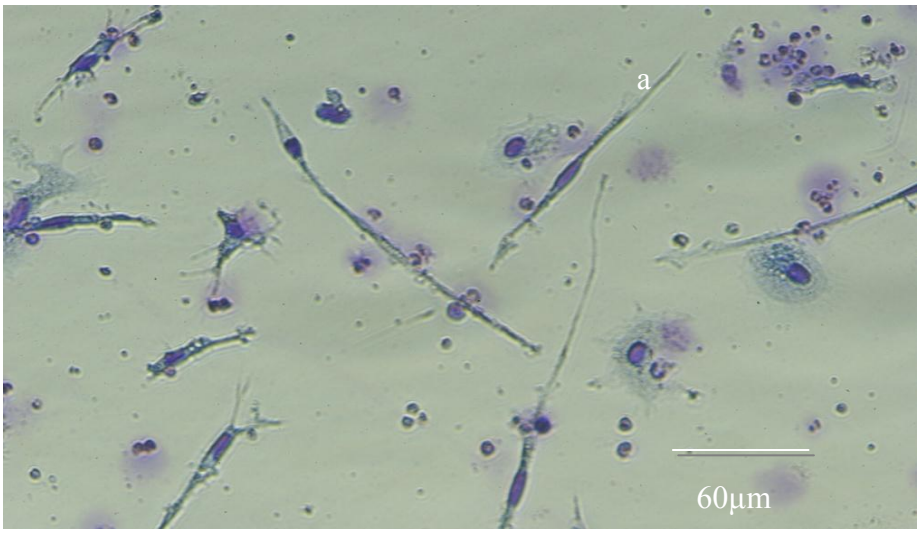

Figure 2: **White blood cells dyed with eosin and methylene blue.** "a" indicates fibrocyte.

SAP

Serum Amyloid Protein P (SAP) circulates in the blood and inhibits the differentiation of white blood cells into fibrocytes [8]. While SAP levels in the blood are at normal levels, around 32 $\pm$ 7 µg/mL for men and 24 $\pm$ 7 µg/mL for women [8], normal wound healing and related functions can occur. When those levels begin to decrease or the activity of SAP declines, fibrocytes become more prevalent and can cause damage to internal processes or diseases like fibrosis. The exact mechanism of how SAP inhibits fibrocyte differentiation has not been determined.

The determination of what compounds affect the number of fibrocytes that develop, and the effect those compounds have on the functioning of SAP are both important concepts to investigate. Finding an explanation for scar tissue formation could lead to improved

treatment options which would decrease fibrosis seen in patients receiving PD. These alternative treatments would save many lives.

CHAPTER II

MATERIALS AND METHODS

Cell culture and fibrocyte differentiation assay

We collected blood from voluntary adult donors in agreement with Texas A&M University's Institutional Review Board. We isolated peripheral blood mononuclear cells (PBMC) as previously described by Pilling et al. [11].

PD fluid contains the following concentrations of ions: 132 mM sodium, 96 mM chloride, 3.5 mM calcium, 0.5 mM magnesium, 40 mM lactate, and 1.5% dextrose. I added these concentrations to water to make PD fluid. I also made solutions of sodium chloride, calcium chloride, magnesium chloride, sodium lactate, and dextrose to test the individual components.

I cultured PBMC in Falcon 96-well cell culture plates to test the effect that varying concentrations of peritoneal dialysis fluid and the separate components had on the differentiation of fibrocytes in RPMI medium. For PD fluid, I added 200μL of PD fluid in the first well followed by 100μL of RPMI in the following seven wells. For the individual salts, I added 200μL RPMI and twice the concentration of salt found in PD fluid into the first well and 100μL RPMI to the following seven wells. I then used serial dilutions to create a range of concentrations by transferring 100μL from one well to next and disposing of the last 100μL. I used two rows of serial dilutions for each salt tested,

and each plate has a row of RPMI with no added salt as a control. 50,000 cells in 100µL of RPMI were added to each well, halving the concentration in the well by doubling the volume. Cells were incubated for five days at 5% CO_2 and 37°C. Following the incubation period, I fixed cells to the plate for ten minutes using methanol and then stained cells for ninety seconds with each of the two dyes eosin and methylene blue.

I counted fibrocytes in five different 900-µm fields of view. My analysis was done using the percent of fibrocytes found in the experimental wells relative to the average of the control wells (RPMI medium with no added salts). I performed t-tests using GraphPad Prism software to determine the statistical significance of increased fibrocyte numbers compared to control.

SAP dilution

I performed SAP dilutions ranging from 5µg/mL to 0.04µg/mL in RPMI medium, 12.5% PD fluid in RPMI, 12.5mM sodium chloride added to RPMI, and 5mM sodium lactate added to RPMI. I used the same PBMC isolation and assay procedure as before. I compared SAP dilution curves of the salts to that of the RPMI medium alone, and with those curves, I found and compared each condition's IC 50 value. These values signify the concentration of SAP which causes 50% differentiation of fibrocytes. I performed t-tests using GraphPad Prism software to determine the statistical significance between IC50 values.

Immunohistochemistry

I used immunohistochemistry to stain for immunological markers on the white blood cells as previously described [11]. I used this staining to verify that the cells I found were fibrocytes. I stained samples with mouse and rat antibodies specific for the cell markers CD 45, CD 68, procollagen, and prolyl-4-hydroxylase.

CHAPTER III

RESULTS

Component salts effect on differentiation

Products of glucose degradation which form during sterilization of glucose solutions can cause an increased development of scar tissue [6]. I wanted to determine if peritoneal dialysis fluid affects the differentiaiton of PBMC into fibrocytes. I found that PD fluid caused a statistically significant ($p<0.001$) increase around 12.5% in the 200 µl *in vitro* cell cultures (Figure 3). Percentages above 12.5% caused cell death.

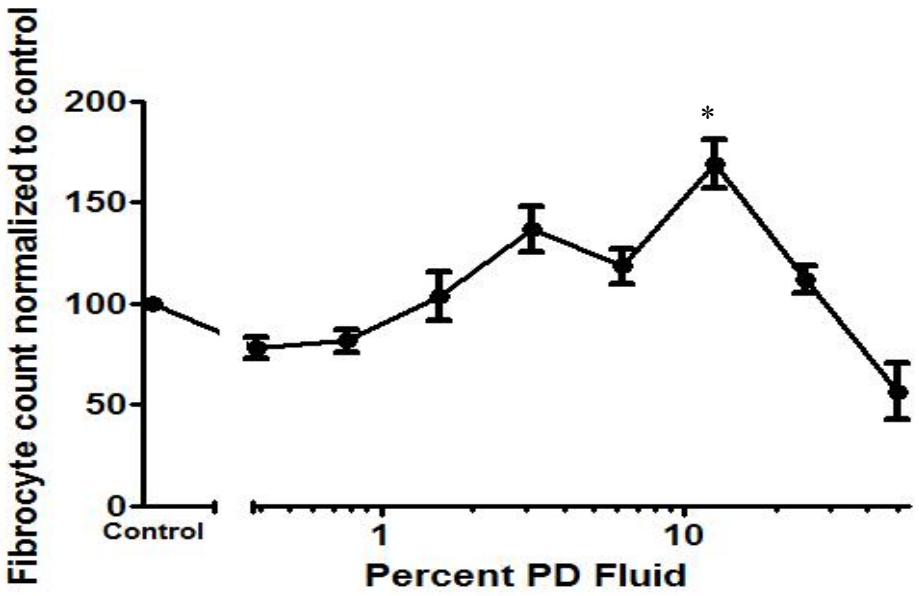

Figure 3: **PD fluid potentiates the differentiation of fibrocytes from white blood cells.** White blood cells were incubated with the indicated concentrations of PD fluid for 5 days. The cells were then stained and counted. Values are mean ± SEM, n=6. 12.5% fluid showed the largest increase in fibrocyte differentiation when compared to the control. This increase was statistically significant (* indicates statistical significance, $p<0.001$). This x-axis is in a logarithmic scale.

To determine whether specific components of PD fluid cause the increase in fibrocyte differentiation caused by whole PD fluid, I cultured PBMC in individual compounds of the dialysis fluid (sodium chloride, sodium lactate, magnesium chloride, calcium chloride, and dextrose). The fibrocyte differentiation increase seen at 12.5 mM sodium chloride (Figure 4) and 5 mM sodium lactate (Figure 5) are statistically significant ($p<0.001$). These concentrations correlate to the 12.5% conventional PD fluid that increased fibrocyte differentiation. Calcium chloride (Figure 6), magnesium chloride (Figure 7), and dextrose (Figure 8) showed no statistically significant increases ($p>0.05$). Upper testing limits for sodium chloride, sodium lactate, calcium chloride and dextrose were dictated both by concentrations found in the PD fluid and concentrations above which cells died. Previous research performed by Katayoon Keyhanian showed similar results with slightly different conditions.

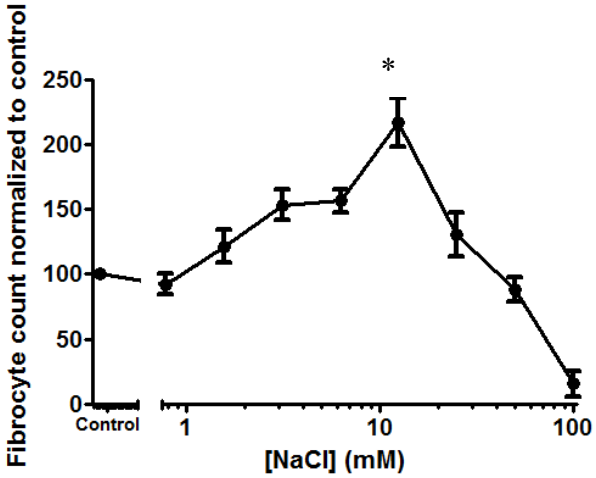

Figure 4: **Sodium chloride causes a statistically significant increase in fibrocyte differentiation.** White blood cells were incubated with the indicated concentrations of sodium chloride for 5 days, after which cells were stained and counted. Values are mean ± SEM n=3. Sodium chloride caused a statistically significant ($p<0.001$) increase of fibrocyte numbers at 12.5 mM (peak indicated by *). This x-axis is in a logarithmic scale.

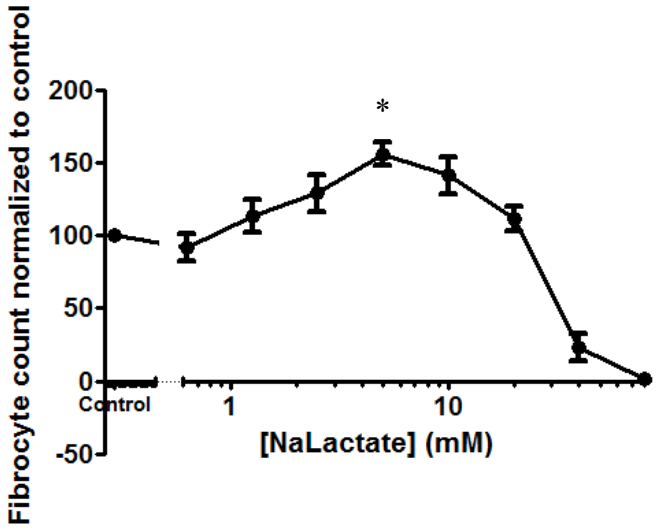

Figure 5: **Sodium lactate causes a statistically significant increase in fibrocyte differentiation.** White blood cells were incubated with the indicated concentrations of sodium lactate for 5 days, after which cells were stained and counted. Values are mean ± SEM n=3. Sodium lactate caused a statistically significant ($p<0.001$) increase of fibrocyte umbers at 5 mM (peak indicated by *). This x-axis is in a logarithmic scale.

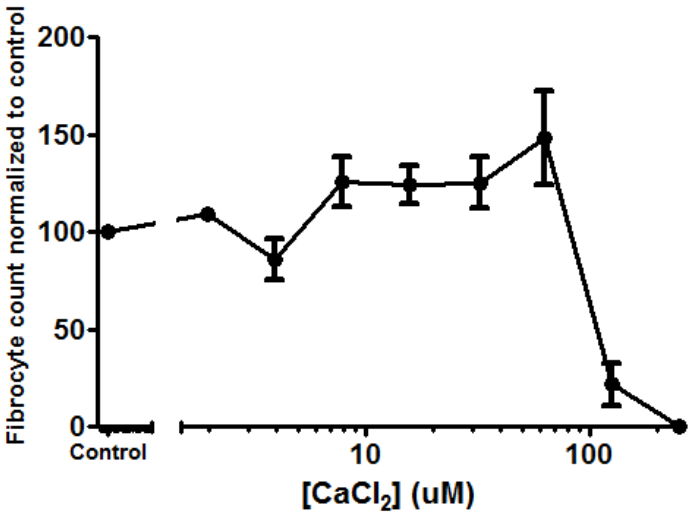

Figure 6: **Calcium chloride causes no statistically significant increases in fibrocyte differentiation.** White blood cells were incubated with the indicated concentrations of calcium chloride for 5 days, after which cells were stained and counted. Values are mean ± SEM n=3. Calcium chloride caused no statistically significant increases in fibrocyte differentiation. This x-axis is in a logarithmic scale. The absence of an error bar indicates that the error was smaller than the plot symbol.

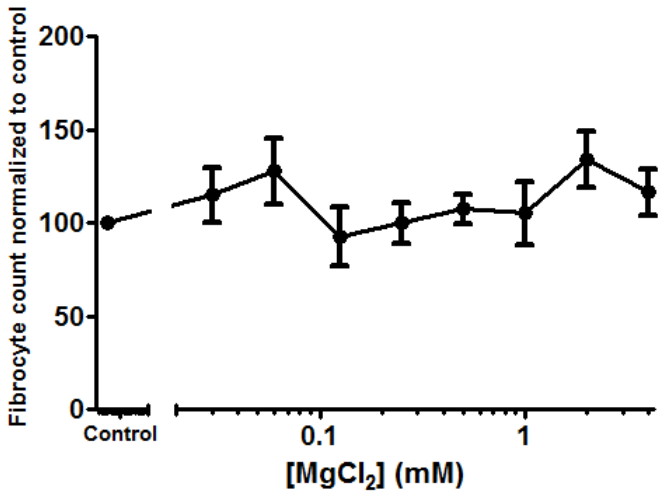

Figure 7: **Magnesium chloride causes no statistically significant increases in fibrocyte differentiation.** White blood cells were incubated with the indicated concentrations of magnesium chloride for 5 days, after which cells were stained and counted. Values are mean ± SEM n=3. Magnesium chloride caused no statistically significant increases in fibrocyte differentiation. This x-axis is in a logarithmic scale.

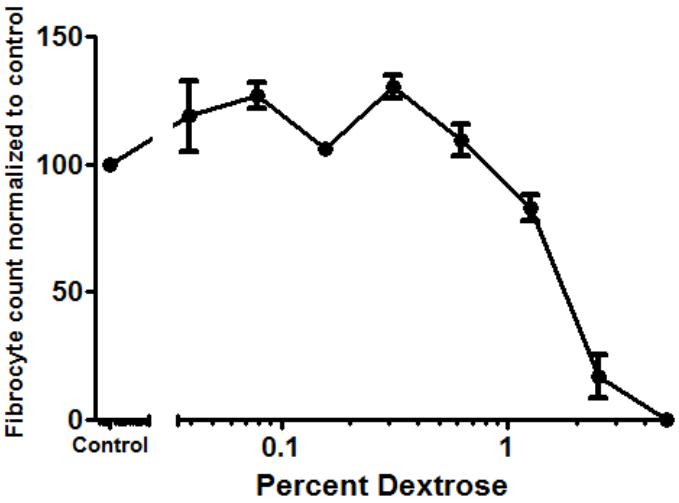

Figure 8: **Dextrose causes no statistically significant increases in fibrocyte differentiation.** White blood cells were incubated with the indicated concentrations of dextrose for 5 days, after which cells were stained and counted. Values are mean ± SEM n=3. Dextrose caused no statistically significant increases in fibrocyte differentiation. This x-axis is in a logarithmic scale. The absence of an error bar indicates that the error was smaller than the plot symbol.

Effects of component salts on the sensitivity of SAP

SAP inhibits fibrocyte differentiation. I sought to determine if salts found in PD fluid lower the effectiveness of SAP by desensitizing the PBMC to SAP. The desensitization would further enhance the formation of fibrocytes. I used the concentrations of salts I found to have the largest effect on fibrocyte differentiation to test the effect they had on the activity of SAP.

IC50 values tell the concentration at which you observe 50% differentiation of fibrocytes. An increase in IC50 value compared to the control signifies desensitization. I observed that PD fluid, sodium chloride, and sodium lactate all decreased the ability of SAP to inhibit fibrocyte differentiation (Figure 9). PD fluid and sodium lactate both had statistically significant increases of the SAP IC50 (Table 1).

Solution	IC50 (mean ± SEM)
Control	0.13±0.03 μg/ml
Peritoneal Dialysis Fluid (PD)	0.31±0.03 μg/ml
Sodium Chloride	0.26±0.05 μg/ml
Sodium Lactate	0.32±0.06 μg/ml

Table 1: **IC50 Values of PD fluid, sodium chloride and sodium lactate.** Increase in IC50 value compared to control indicates desensitization. Statistically significant increases were observed for PD fluid and sodium lactate ($p<0.05$).

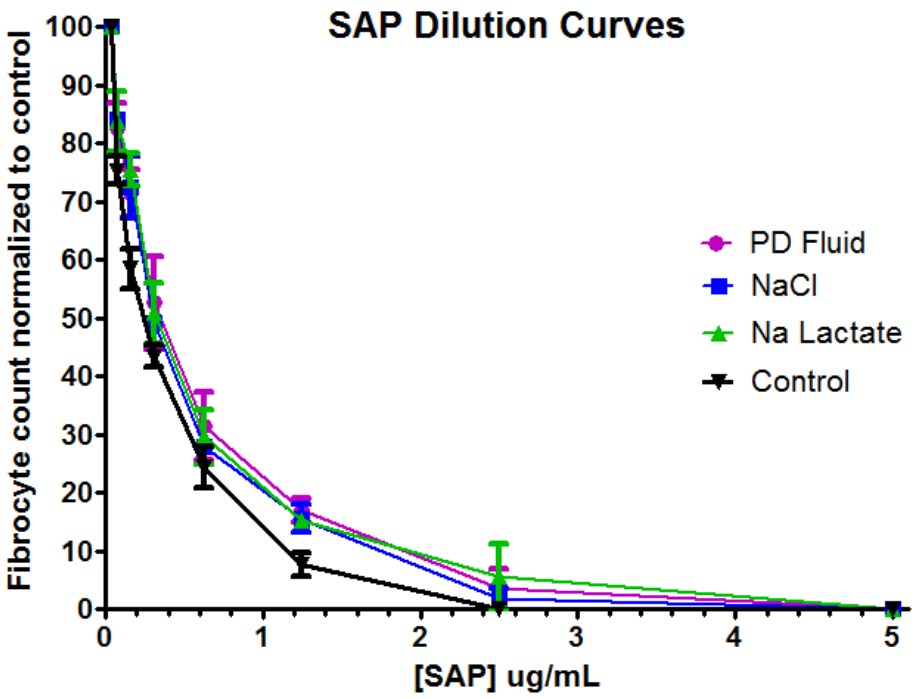

Figure 9: **PD fluid, NaCl, and Na lactate desensitize the white blood cells to the effects of SAP.** The response of white blood cells to increasing concentrations of SAP was investigated in the presence of PD fluid, NaCl, and Na lactate. All the added solutions caused a decrease in the sensitivity of white blood cells to SAP. I found that whole PD fluid, sodium chloride, and sodium lactate decreased the ability of SAP to inhibit the development of fibrocytes.

Immunohistochemistry

Fibrocytes display antibody markers for both stromal and hematopoietic cells. To certfiy that I found fibrocytes, I stained the cultured cells using antibodies that recognize fibrocyte markers. Staining for markers CD 45, CD 68, Prolyly-4-hydroxylase, and procollagen showed positive results, indicating that the cells that I counted as fibrocytes were indeed fibrocytes.

CHAPTER IV

SUMMARY AND CONCLUSIONS

I found that dialysis fluid and its component salts sodium chloride and sodium lactate caused significant increase in human fibrocyte formation in tissue culture compared to the control (RPMI). All of these statistically significant increases occurred around 12.5% of the concentrations found in whole fluid. This increase in fibrocyte formation could provide one explanation for scar tissue development in the abdominal cavity.

I then tested the effect of PD fluid and its components on the ability of SAP to inhibit fibrocyte differentiation. IC50 values tell the concentration at which you observe 50% differentiation of fibrocytes. The increase in IC50 values of the salts compared to that of the control indicates a desensitization of the white blood cells to SAP. This desensitization of PBMC to SAP provides a second explanation for why PD fluid potentiates scar tissue formation.

These results show that components of PD fluid enhance the development of fibrocytes. It accomplishes this both by promoting the development of fibrocytes and desensitizing cells to the inhibiting protein SAP. Together, these results could provide an explanation for the development of peritoneal scar tissue during dialysis treatment.

In the future, research could focus on how to change the composition of the dialysis fluid to decrease the development of fibrocytes. Different salts or fluid composition could reduce the risk of fibrosis development in patients receiving PD.

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